Title: To investigate the relationship between energy homeostasis and insulin resistance
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INTRODUCTION

Accumulation of excess lipid or fat is known to be associated with metabolic diseases together known as metabolic syndrome. This is steadily increasing in global scenario for last three decades. Diabetes mellitus is one of such major disease. Among diabetic patients 3-5% are type 1 diabetes (T1D) and 95-97% patients are Type 2 diabetes(T2D), T2D is the global epidemic disease [1]. One of the important reasons for T2D is defects in function of insulin on its target tissues specially skeletal muscle and adipose tissue, a condition called insulin resistance. 75% of postprandial (PP) sugar is deposited in muscle where as only marginal sugar is absorbed in other tissues[2], therefore, if defects in skeletal muscle could be improved, incidence of T2D might be possible to reduce. Unfortunately, despite several investigations, it is not clear till date that how muscle gets affected during diabetic condition. On the other hand, the primary reason for insulin resistance is excess lipid induced inflammation, where both adipocyte and macrophage participate. Therefore, at this juncture a question arises that if there is any deregulated inflammatory action or any other reasons behind the attenuated sensitivity of insulin in skeletal muscle.

REVIEW OF LITERATURE

Several report have suggested that oversupply of lipid is one of the major forces behind this insidious disease [3]. However, despite several publications in this area underlying mechanism of excess lipid accumulation and its association with insulin resistance is yet unclear. What are the signalling defects behind this are of the major questions. At this point, it is important to note that adipocytes are the major cells in adipose tissue, there are other cells too, but these are the cells which could uptake

lipid, store them and metabolize them [4]. Interestingly adipocyte morphology varies due to excess lipid deposition; for example, preadipocytes and small adipocytes are seen in normal condition, but hypertrophied and senescent adipocytes increases due to obesity induced abnormalities [5]. Increase in hypertrophied adipocytes coincided with the impairment in the uptake of fat and storage of fatty acids in the form of triglycerides.

One of the dangerous attribution of these defective adipocytes is the creation of imbalance between energy storage, accumulation and expenditure. It is indeed intriguing to note about how intracellular signalling and metabolic pathways respond to various challenges involving distinct cellular entity to permit proper adaptation. Regulation of energy at cellular level, specifically that deals with energy intake, energy storage and energy expenditure, whenever there is a disruption in their balance, a defect occurs in coordinating the energy homeostasis leading to cellular signalling defects [6]. This metabolic defect leads to a cluster of diseases which fall under the category of "metabolic syndrome" that includes diabetes, atherosclerosis, hypertension, other cardiovascular problems, rheumatism, etc. Energy metabolism primarily includes two important food components, one is carbohydrate and other is lipid or fat. Carbohydrate provides more easily available energy, even storage of it cannot sustain the energy requirement for a long time. More important form of energy storage is lipid or fat and it is lipid which is responsible for two important critical diseases i.e diabetes and cardiovascular problems.

Sirtuins are a group of NAD+ dependent histone 1 protein deacetylases, that are regulated by changes in the cellular redox state ($NAD^+/NADH^+$ratio) [7]. When the ratio crosses the threshold, the SIRT1 is activated. In energy deficient condition of a cell the ratio of NAD^+ and $NADH^+$ remains higher than that of a cell in fully fed condition. So the SIRT1 is activated when there is need of energy. After activation, it deacetylates and activates PGC1α. Increased PGC-1α induces the transcription of nuclear respiratory factor NRF1 and NRF2, leading to the increased expression of mitochondrial transcription factor A (mtTFA) as well as other nuclear-encoded mitochondria subunits of the electron transport chain complex such as β-ATP synthase, cytochrome *c*, and cytochrome *c* oxidase [8,9]. mtTFA translocates to the mitochondrion, where it stimulates mitochondrial biogenesis as manifested by

stimulation of mitochondrial DNA replication and mitochondria gene expression [10]. Thus SIRT1 produce energy from the stored food. Interestingly, it is shown that SIRT1 can activate AMPK by deacetylating its activator LKB1,that promotes its translocation from nucleus to cytosol where it in turn phosphorylates AMPK and activates it [11]. Conversely, AMPK can activate SIRT1 by increasing the NAD+/ NADH+ ratio or by expression/activity of NAMPT [12]. All these findings suggest that the existence of an AMPK/SIRT1 cycle that links the cellular redox and energy states. AMPK and SIRT1 act on common transcriptional activators and co-activators including members of FOXO family and PGC1α. In addition, both SIRT1 and AMPK activators has been shown to decrease atherosclerosis and prevent diabetes in experimental animals. Both AMPK and SIRT1 exert effects on inflammation and fuel metabolism in M1 macrophage and T helper cells which are found in huge quantity in inflamed adipose tissue.

KNOWLEDGE GAP

1) Impairment of insulin sensitivity in skeletal muscle in producing T2D is yet unclear, though, 75% of postprandial glucose enters into the muscle where it is stored and being a mitochondria rich tissue it plays the major role in utilizing the stored energy. It has been clearly shown that in diabetic condition or in HFD mice the fatty acid oxidation, mitochondrial biogenesis in muscle is hampered. But how the energy production in muscle is impaired or what are the cellular signals that are involved in this process , if there is any change of expression and activity of the energy sensors are not clearly known.

2) Muscle being the less prone tissue for inflammation, how it gets defected during T2D is a shaded area of research. There is no clear picture about the level of inflammatory proteins and their association with defects in insulin signalling pathway.

3) Adipose tissue inflammation gradually increase with the gradual deterioration of muscle insulin sensitivity. 80% of the total volume of adipocyte is filled with lipid, remaining 15-17% is cytosol. Even with this small amount of cytosol it secretes about 347 proteins of which some are increased and some are decreased during adipose

tissue inflammation. It is interesting that there is a negative regulation of SIRT1 in inflamed adipose tissue, where it is cleaved and gets inactivated and as a result the stored food cannot get converted into energy. This kind of regulation is mainly induced by inflammation. Now what is the status of inflammation or inflammation induced disruption of energy homeostasis in muscle in obese condition is not clear.

OBJECTIVES

1) To observe the changes occurred in the expression and activity of the energy sensors such as SIRT1 and AMPK in skeletal muscle and adipose tissue in the diabetic mice model.

2) To investigate on how excess lipid could induce insulin insensitivity by disrupting energy homeostasis both in muscle and adipose tissue. The negative signals generated during the imbalance of energy homeostasis are also required to be examined.

3) As mentioned above that there is a strong relationship between adipose tissue and skeletal muscle cells regarding excess energy storage which is related to proinflammatory signal induced damage of insulin sensitivity and cause T2D. Examination of this relationship will be studied to understand the nature of crosstalk between these two important metabolic tissues.

METHODOLOGY :

1. Models of study

The study will be conducted in different models as mentioned below

(i) ***Animals*** – Adult normal healthy male BALB/c mice would be conditioned with a 12 h light/12 h dark cycle at 23 ± 2°C and relative humidity 55 ± 5% along with access to standard diet *ad libitum*. A subset of control BALB/c mice would be reared on high-fat-diet (HFD) for 12 weeks to render them insulin resistant.[6,15] Besides, genetically modified strains of mice established as obese and type 2

diabetic models viz. *db/db* mice would also be considered for study. Body fluids and tissues, visceral adipose tissue, muscle, liver would be obtained from the animals. Animal handling, maintenance and experiments would be performed following approval and guidelines prescribed by the Institutional Ethics Committee.

(ii) ***Primary culture*** – Adipocytes would be isolated from animal models and cultured *in vitro* following our earlier method.[6] In brief, adipose tissue would be rinsed with sterile 0.9% NaCl and thoroughly washed in HBSS supplemented with 5.5 mM glucose and digested in HBSS buffer containing 5.5 mM glucose, 5% fatty acid-free BSA, and 3.3 mg ml^{-1} of type II collagenase for 30 min at 37 °C. The digestion mixture would be passed through tissue sieve and adipocytes suspended in serum-free DMEM. Finally the cells would be plated in 6-well culture plates in humidified 5% CO_2 at 37 °C. Skeletal muscle tissue would be cultured as slices in serum-free RPMI in 6-well culture plates in humidified 5% CO_2 at 37 °C as slices.

(iii) ***Cell lines*** – Mice muscle cell line C2C12 and L6, mice 3T3-L1 preadipocytes, would be differentiated using adipogenesis assay kit of Cayman according to manufacturer's protocol before treatment. Mice macrophage cell line RAW 264.7 and human cell lines may also be used for study.

2. Protein estimation

The protein content of tissue extracts, cell lysates and media would be determined by the method of Lowry *et al*.[13]

3. Treatments:

To create an insulin resistant model, the control mice would be fed high-fat-diet (HFD) for 12 weeks as earlier done by us.[6,14] The HFD consists of 32.5% lard, 32.5% corn oil, 20% sucrose, and 15% protein whereas standard diet consists of 57.3% carbohydrate, 18.1% protein, and 4.5% fat. The

energy content of standard diet is 15 kJ/g and HFD is 26 kJ/g. All cell culture treatments would be conducted in serum-starved condition. Treatments with saturated fatty acid such as palmitate, fetuin-A, resveratrol (SIRT1 activator), sirtinol (SIRT1 inhibitor), several inhibitors (PDTC, SN50, SP600125, CLI-095) and other chemicals would be carried out as described.[6,13] Various short-interfering siRNA and vector transfections would be performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

3. 2-deoxyglucose uptake

Isolated adipocytes and skeletal muscle cells would be incubated in Krebs-Ringer phosphate buffer supplemented with 0.2% BSA followed by 30 min of incubation in the presence of insulin (100 nM). [^{3}H]-2-deoxyglucose (0.4 nmol/ml) would be added to each incubation 5 min before the termination of incubation. Cells would then be washed three times with ice-cold Krebs buffer in the presence of 0.3 mM phloretin. The cells would be solubilized with 1% NP-40 and [^{3}H]-2-deoxyglucose would be measured in liquid scintillation counter.[6]

4. [^{14}C]-Palmitate uptake

Adipocytes and muscle without or with fetuin-A would be incubated with 1 μCi/mol [^{14}C]-Palmitate for 3 h. Cells would be washed three times with ice-cold Krebs-Ringer phospahe buffer. Cells would be solubilized with 1% NP-40 and [^{14}C]-palmitate would be measured in liquid scintillation counter.[15]

5. Transwell system of co-culture

In order to simulate the microenvironment that exists during inflammatory condition, indirect co-culture would be performed *in vitro* using transwell system (Thermo Nunc). Differentiated 3T3L1 adipocytes would be seeded on the bottom whereas C2C12 or L6 would be cultured onto the membrane of transwell cell culture inserts. Co-cultures would be treated as required and the media and/or cell lysates would then be finally analyzed for gene/protein expressions.

6. Cell migration assay

Cell migration assay would be performed in Boyden chamber system (Millipore QCM 24-well colorimetric cell migration assay kit).[15] Addition of fetuin-A, and other chemoattractants and cytokines/chemokines would be done in the lower chamber. After the required period of incubation, the cells would be stained and photographed.[15] The absorbance would be then measured in 96-well microplate reader.

7. ChIP assay

Chromatin Immunoprecipitation would be performed by using ChIP assay kit (Upstate) in human/mice adipocytes and muscle. PCR products would be resolved in ethidium bromide-stained 1.5% agarose gel, and image captured in Bio-Rad gel documentation system.

8. Immunofluorescence

Paraformaldehyde or chilled methanol fixed cells would be incubated with respective primary antibodies followed by incubation with fluorescence conjugated secondary antibody for 2 h. Cells would be mounted in DAPI mounting medium for nuclear staining if required. Immunofluorescence would be seen with a fluorescence or confocal microscope.

9. Immunohistochemistry

The adipose tissue and miscle will be isolated from both the control and HFD groups. The tissue will be fixed in Bouin's fluid followed by dehydration in ascending grades of alcohol. Paraffin embedded tissues will be cut at appropriate thickness using a Microtome and affixed on glass slides for further observation. The sections will be deparaffinised in xylene and rehydrated with descending grades of alcohol. For

immunohistochemical labeling, tissue sections will be sequentially treated with blocking solution (1–2 h), incubated in primary antibodies overnight (4°C), and incubated with appropriate Alexa fluor-conjugated secondary antibodies (1–2 h) the following day. Draq5 (Biostatus Limited) at 1:3000 dilution will be used as a nuclear counterstain. Immunofluorescently labeled M1 and M2 cells will be quantified under high-power magnification with wide field-fluorescence(Lumeng et al., 2007). Macrophage specificity will be confirmed by double labelling with macrophage markers (tomato lectin) and M1 or M2 markers (TNFα, IL-6, MCP-1 and PPARγ, 1L-10 respectively).[16]

10. Immunoblotting

Protein content (50 μg) from tissue extracts, cell lysates and media would be resolved by SDS-PAGE and then transferred on to PVDF membranes (Millipore) by wet transfer or Semi-Dry method using Trans-Blot® Apparatus (Bio-Rad Laboratories). The membranes would be then blocked by skimmed milk and incubated with primary antibodies followed by secondary antibody conjugated to alkaline phosphatase. Protein bands would be detected by using BCIP (5-bromo-4-chloroindol-3-yl phosphate)/NBT (nitro blue tetrazolium).

11. Reverse-transcription PCR (RT-PCR) and qPCR

Total RNA would be extracted from different incubations using TRI Reagent (Sigma) or RNeasy Lipid Tissue Mini Kit (Qiagen) according to manufacturer's instructions. RT-PCR would be carried out using first–strand cDNA synthesis kit (Fermentas). Gene expression would be further validated by SYBR green based quantitative real-time PCR (Applied Biosystems) by using gene-specific primers. The housekeeping gene, *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) would be simultaneously amplified in separate reactions for correcting the C_t value.

12. ELISA

Various proinflammatory markers (TNFα, IL-6, IL-1β etc.) and anti-inflammatory markers (IL-10 etc.), adiponectin, SIRT1, fetuin-A and other signaling molecules would be measured in cell culture media or lysates or tissue extracts by enzyme-linked immunosorbent assay using ELISA kits (Ray Biotech) according to manufacturer's instructions.

13. FACS

M1 macrophages would be labeled using CD11c antibody (BD Pharmingen) or with their isotype control (Santacruz Biotechnology) for 30 min at 4°C. After washing with PBS, the labeled cells would be analyzed by flow cytometry using a BD FACSAria™ II and FACS Diva Software. Similarly, macrophages would be labeled for other cell surface markers.

14. Isolation of Mitochondria

First the tissues and cells would be homogenized in a buffer containing 225mM mannitol, 75mM sucrose, 5mM HEPES, 1mM EGTA, and 1mg/ml BSA and then would be centrifuged at 2000g for 5 min at 4°C. Then the super natant will be aspirated out and again centrifuged at 12,000g for 10 min and the brown mitochondrial pellet formed would be washed twice with 10ml of washing buffer (225 mM mannitol, 75 mM sucrose, and 5 mM HEPES pH 7.4) at 12,000g for 10 min at 4°C and finally resuspended in 50 mM potassium phosphate buffer and stored at 20ºC for subsequent enzyme assays. The mitochondrial complex activities would be nmeasured within 48 h following an earlier published method. For carrying out measurement of mitochondrial ATP synthesis, the freshly obtained mitochondrial pellet was resuspended in isotonic buffer B containing 145mM KCl, 50mM sucrose, 1Mm EGTA, 1mM magnesium

chloride, 10 mM phosphate buffer, pH 7.4. For estimation of mitochondrial DNA (mtDNA) copy number, first total DNA would be extracted from cells and tissues and then the content of mtDNA calculated using real time quantitative PCR by measuring the threshold cycle ratio (ΔCt) of a mitochondrial encoded gene COXII versus a nuclear encoded gene RIP140 as described earlier.

15. Statistical analysis

Data will be analyzed by one way –analysis of variance (ANOVA) where the F- Value indicates significance, means will be vcopaired by a post hoc multiple range test. All values will be represented as mean ± SEM taking P <0.05 as threshold of significance.

16. Keywords

Insulin resistance, energy homeostasis, energy sensors, sirtuin-1, type 2 diabetes, adipose tissue, skeletal muscle, inflammation

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